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17 The protein epitope mimetic approach to protein-protein interaction inhibitors

John A. Robinson and Kerstin Moehle

17.1 Introduction

The design of small-molecule protein epitope mimetics is now widely recognized as a promising structure/mechanism-based approach to the discovery of protein-protein interaction (PPI) inhibitors. Progress in the field has been driven, on the one hand, by the enormous increase in the knowledge base of PPIs, arising from high throughput genomic and proteomic methods (Lehne and Schlitt 2009; Stumpf et al. 2008), and on the other, by the massive growth in the 3D structural database of protein-protein complexes. The key scientific challenge is to convert this structural and functional information, through a rational molecular design process, into new PPI inhibitors with potential value in chemical biology, or in drug and vaccine discovery. Ideally, structural information on PPIs should be combined with an understanding of the mechanism(s) of PPIs, to aid the design process. In this review article, we begin with a short overview of recent mechanistic studies of PPIs. Following this, an overview is provided of methods now available for the discovery of PPI inhibitors, in particular, based on the protein epitope mimetic approach.

17.2 Mechanisms of protein-protein interactions

Protein-protein interfaces are often quite large (in the range 600 to ~2,500 Å^2 on each side) (Conte et al. 1999; Jones and Thornton 1996), and although they are never really flat, they do lack well-defined binding pockets of the type characteristic of enzyme active sites. It is probably worthwhile to differentiate between inhibitors targeting enzyme active sites and those directed toward PPIs. Enzymes have evolved to catalyze chemical reactions, mostly by binding tightly to the reaction transition state (TS). In other words, the required complementarity between the molecule undergoing reaction and the packing within the protein are most efficient in the enzyme-TS complex. Based upon comparisons of reaction rates in enzyme catalyzed and noncatalyzed processes, it has been estimated that enzyme-TS complexes typically have dissociation constants in the range 10^{-12} M to 10^{-20} M (average $10^{-16\pm4}$ M), which is much greater than expected from the surface areas of TSs (Zhang and Houk 2005), in particular for those enzymes that operate by noncovalent interactions with their substrate(s) (Smith et al. 2009). Many active site-directed enzyme inhibitors act as structural analogues of the TS, and in optimal cases may achieve similar levels of complementarity and affinity with the enzyme. The complementarity between the protein and TS typically includes a network of interactions extending into the protein shell surrounding the active site. This view is supported by recent studies with the enzyme purine nucleoside phosphorylase, which provided evidence for conformational collapse in the protein around a potent inhibitor in the active site due to its structural similarity to the TS (Edwards et al. 2010). Thus, with enzymes, improved packing and positive cooperativity in binding to the TS architecture

may involve an extensive network of interactions, perhaps extending some way into the protein shell around the active site (Williams 2010).

For many PPIs, average dissociation constants tend to be in the micromolar to nanomolar range, not because higher affinities are impossible, but because in many cases no strong evolutionary pressure exists for further affinity maturation, as pointed out already for the case of antibody-antigen interactions (Foote and Eisen 1995). The theoretical diffusion-limited association rate for two molecules in solution is likely to be around 5×10^9 M^{-1} s^{-1} (the Einstein-Smoluchowski limit [Berg and von Hippel 1985]). Many PPIs in solution have association rates that are slower (in the range 10^4–10^7 M^{-1} s^{-1} (Harel et al. 2007; Karlsson et al. 1991; Raman et al. 1992), although electrostatic steering effects can lead to substantially faster association rates (Gabdoulline and Wade 2002; Schreiber 2002; Schreiber and Fersht 1993, 1996; Selzer and Schreiber 1999). A minimalist three-state model for protein-protein association was suggested, based on Brownian dynamics simulations, with a first rapid diffusion controlled formation of an encounter complex, in which a protein pair surrounded and trapped by a solvent cage undergoes multiple collisions and rotational reorientation during each encounter (Gabdoulline and Wade 2002; Northrup and Erickson 1992; Northrup et al. 1988; Selzer and Schreiber 2001; Tang et al. 2006). In a second rate-determining step, the two proteins rearrange to the final stereospecific bound state, which might be rate-limited by conformational transitions and desolvation steps, amongst other things (Harel et al. 2007). Assuming that a lifetime for a protein-protein complex beyond 20–30 min would (in many cases) bring little significant extra biological advantage, an effective limit for the off-rate can be fixed at 10^{-3} to 10^{-4} s^{-1}, which would in turn give a ceiling on affinity around 10^{-9} to 10^{-10} M. Enzyme inhibitor design based on mimicry of TSs may, in principle, tap-into a higher potential binding energy (average K_d $10^{-16\pm4}$ M) focused at an active site whose architecture is often complementary to that of a small-molecule ligand.

The cores of monomeric proteins are stabilized consistently by hydrophobic interactions. At some protein interfaces, hydrophobic forces play an important role (e.g. human growth hormone [hGH]-hGH-receptor [hGHR] complex]Wells 1996]), but in others mainly polar or even charge-charge interactions seem to be dominant (e.g. barnase-barstar) (Tsai et al. 1996, 1997). There appears to be no significant difference in the chemistry or geometry of individual interactions at protein interfaces compared to the cores of folded monomers. However, side-chain-to-side-chain interactions are more frequent at protein interfaces compared to protein cores (Cohen et al. 2008). A number of studies have pointed out that energetically important residues at interfaces (hot spots – ▶Figure 17.2, further on in this chapter) are enriched particularly in tryptophan, tyrosine and arginine, while some other amino acids are found very rarely in hot-spots (Val, Leu, Ser, Thr) (Bogan and Thorn 1998; Hu et al. 2000; Ma et al. 2003; Padlan 1990). The preference for Trp, Tyr and Arg may be due, at least in part, to the ability of these side-chains to engage simultaneously in several types of favorable interactions, which in turn facilitate the creation of interaction networks at protein interfaces (see further on in this section). Recently, minimalist combinatorial libraries based on restricted amino acid usage have been used to identify synthetic protein interfaces (Kossiakoff and Koide 2008). For example, interfaces built using just two different amino acids (Tyr/Ser) in the context of antibody CDR loops are sufficient to produce antibody fragments with high affinity and specificity for protein targets (Gilbreth et al. 2008).

Alanine-scanning mutagenesis led in 1995 to the identification of so-called hot-residues located within "hot-spots" that have since been found in many protein-protein interfaces (Clackson and Wells 1995; Clackson et al. 1998; Moreira et al. 2007; Schreiber and Fersht 1995). Ala mutations that reduce affinity by >10-fold indicate a hot-residue making a disproportionately large contribution to the binding energy. Such hot residues often cluster at the center of interfaces, constitute less than half of the contact surface, and are surrounded by other residues, which by this definition, contribute relatively little in binding energy (DeLano 2002; Keskin et al. 2005; Reichmann et al. 2007). Not surprisingly, hot spots have become a major focus of interest in PPI inhibitor design (Wells and McClendon 2007). One mechanistic interpretation of hot-spots is that the peripheral residues serve as an O-ring to exclude solvent from the center, which would generate a lower effective dielectric and strengthen hydrogen-bonding and other electrostatic interactions (Bogan and Thorn 1998). Similar effects are known to be important in enzyme catalysis, where water exclusion from the active site is often important for catalytic efficiency. However, an alternative view has been proposed, where an interaction deleted by Ala mutagenesis in the periphery might be more easily compensated by a bridging water molecule, and hence causes less loss in stability (Janin 1999). From structural studies it is known that protein interfaces contain on average more bound water molecules than are found in the core of a folded protein. Modeling studies have suggested that both effects may be operative in some cases (Kortemme and Baker 2002). In one mutagenesis study, it was possible to improve the affinity of a PPI from a K_d of 23 nM to 0.15 nM through mutations in a V_H domain interacting with its protein antigen (Koide et al. 2007). In this case, a crystallographic study suggested that the mutations did not expand the antibody-antigen interface, but rather induced an extended network of interactions, and included additional hot residues at the periphery (not the center) of the epitope. These results indicate that although the O-ring architecture may occur frequently, it is not a prerequisite for high affinity PPIs.

More recently, double mutant cycle analyses with selected PPIs have suggested that interfaces between proteins are often built in a modular fashion. Double mutant cycles may reveal whether the contributions from a pair of mutated residues are additive or whether the energetic effects are coupled through neighboring residues (Albeck et al. 2000). In this way, the interface between TEM1-ß-lactamase and ß-lactamase inhibitor protein (K_D 1 nM) was shown to have a modular architecture made up of clusters of residues with strong intracluster connections and weak intercluster connections (Reichmann et al. 2005). The individual residue clusters appeared to be largely independent of each other energetically. A high degree of cooperativity within each cluster was apparent, to the point that in one case the deletion of an entire cluster had little impact on the structure of the interface, whereas single mutations within a cluster could lead to structural rearrangements of their cluster (Potapov et al. 2008). Computational analyses have also revealed that hot-spot residues may be clustered locally within tightly packed interface regions, and that within these relatively dense clusters, the residues form a dense network of interactions, suggesting that their contributions to the stability of the complex should be cooperative (Halperin et al. 2004; Keskin et al. 2005). A comparison of four different PPIs, with affinities ranging from 2 µM (CheA-CheY complex) to 0.01 pM (barnase-barstar complex) suggested that the extent of connectivity within a cluster of interactions, rather than the size of the interface per se, might be the driving force behind tight binding. The degree

of connectivity was found to be much higher in the barnase-barstar complex than in the other three analyzed. The CheA-CheY interface is characterized by relatively few contacts between the proteins and only one medium-sized cluster, whereas the barnase-barstar interface has two clusters, one cluster being highly developed and forming a very elaborate network of interactions. In the barnase-barstar interaction (Schreiber and Fersht 1993), the high affinity (K_d 0.01 pM) and electrostatically driven fast rate of association (k_{on} 6×10^8 s^{-1} M^{-1}) are likely a response to the strong evolutionary pressure on the cell (*Bacillus amyloliquefaciens*) to develop a potent inhibitor (barstar) of the RNase (barnase), to keep the deadly RNase in an inactive form until it can be exported from the cell. This strong selection pressure is apparently able to overcome the inherent difficulty in constructing the high level of organization in the network of interactions seen at this protein interface (Reichmann et al. 2005). These results highlight the importance of higher network organization within protein interfaces, and not just of surface complementary principle, in accounting for binding affinity and specificity. This network or cluster view of protein-protein interactions has important implications for inhibitor design, although the focus is often on designing molecules to fill pockets ("knobs-into-holes") on the surface of proteins.

Finally, it is important to consider the key role played by water molecules in PPIs. Several surveys have shown that water is far more abundant at interfaces, than in the cores of folded proteins (Conte et al. 1999). Water-mediated polar interactions are as abundant at interfaces as direct protein–protein hydrogen bonds, and they may contribute significantly to the stability of the assembly (Ikura et al. 2004; Rodier et al. 2005). Uncertainties in the location and role of water molecules at PPIs contribute greatly to the difficulties in calculating binding affinities by computational methods (Wang et al. 2011), and in attempts to interpret thermodynamic data (binding enthalpy/entropy) in structural terms.

Binding driven by association of hydrophobic surfaces is conventionally viewed as being driven by favorable entropic contributions, due to release of surface-associated water molecules to the bulk solvent (Chandler 2005; Dunitz 1994). However, the generality of this view has recently been challenged in a simple model system comprising a spherical hydrophobic ligand binding to a solvated hemispherical cavity (Baron et al. 2010; Setny et al. 2010). According to MD simulations, the free energy of this binding process is not dominated by the direct interaction between the ligand and pocket, but by the contributions of water. Contrary to expectation, in this model system the binding is driven by enthalpy and opposed by entropy. This was explained by the release of water molecules from the pocket, and the loss of entropy as arising from the expulsion of disorganized water from the receptor cavity (i.e. cavity water is more entropic than bulk water). An interesting experimental study of protein-ligand interactions revealed just such a thermodynamic profile; an increasingly favorable enthalpy and increasingly unfavorable entropy as the hydrophobic surface area of the ligand increases (Homans 2007; Malham et al. 2005). Another recent survey of protein-ligand interactions found that for the majority of small-molecule interactions with proteins, the enthalpy change provides the largest favorable contribution to binding, and contrary to expectation the trend to enhanced affinity with greater burial of apolar surface is, in general, only weakly correlated with a favorable entropy change (Olsson et al. 2008).

17.3 Small-molecule screening approaches

High-throughput screening has often not been very successful for identifying PPI inhibitors (Arkin and Wells 2004; Fry 2006; Toogood 2002). One explanation for this poor performance might be that the large compound libraries typically used for screening, contain molecules of insufficient (or inappropriate) structural diversity. For example, relatively small, flat (sp^2 hybridized), aromatic or heteroaromatic compounds, might be overrepresented in libraries, because of their perceived likelihood to interact with enzyme active sites or G-coupled protein receptors (GPCRs). Mechanistic considerations, however (see Section 17.2), suggest that such molecules might not be well suited in the search for PPI inhibitors. So what types of property should an ideal screening library have? This question was addressed in a recent study in which approximately 15,000 compounds from three different sources (commercial libraries, academic diversity-oriented synthesis projects, and natural products) were shown to have quite different properties, when tested for their ability to bind a collection of unrelated proteins (Clemons et al. 2010). The study showed that increasing the content of sp^3-hybridized and stereogenic centers, relative to compounds from commercial sources, improved protein binding selectivity and frequency. Not surprisingly, the compounds of highest stereochemical complexity were most often those from natural sources, although continuing improvements in organic synthesis methodology (e.g. diversity oriented synthesis) are also making such molecules increasingly accessible. The structural and stereochemical complexity of natural products relative to small drug-like molecules, and their value in the drug-discovery screening exercise, continue to be well argued (Ganesan 2008; Harvey 2008; Li and Vederas 2009). It is worth noting that many stereochemically complex natural products are macrocyclic compounds in the molecular weight range 0.5–2 kDa. Molecules of this size and shape occupy an area of molecular space that nature has obviously explored (at least to some extent), but which due to its shear size is much more difficult to explore thoroughly using diversity-oriented synthesis.

The so-called fragment-based approach is now also being applied to the discovery of PPI inhibitors (Coyne et al. 2010; Erlanson 2006; Murray and Blundell 2010). The goal here is to build drug leads by identifying small molecular fragments that bind to adjacent sites on the protein with μM or even mM affinity, and then either linking or expanding them to improve affinity and selectivity. The first practical demonstration of the approach used SAR by NMR to identify and link small fragments (200–300 Da) that bind to FK506-binding protein (Shuker et al. 1996). A related idea called "tethering" aims to discover weakly binding ligands through an intermediary disulfide tether (Erlanson et al. 2000). A native or engineered cysteine in a protein is allowed to react reversibly with a library of disulfide-containing small molecules. The cysteine-captured ligands are then identified by MS.

A more recent example of the fragment-based approach is the discovery of inhibitors of Bcl-X_L, which are important targets in anticancer therapy. The protein Bak adopts an amphipathic α helix that binds to Bcl-X_L through hydrophobic and electrostatic interactions (crystal structure, PDB 1BXL) (Sattler et al. 1997). Two small-molecule hits that target the groove-like binding site on Bcl-X_L were found using SAR-by-NMR. These were linked and the resulting ligand was optimized in several steps to give ABT-737, with subnanomolar affinity for Bcl-X_L (Oltersdorf et al. 2005). A related compound ABT-263 proved to be orally bioavailable, and advanced into clinical testing in cancer (Park et al.

2008). Interestingly, 3D structures of Bcl-X_L bound to two low affinity starting fragments (PDB 1YSG), and bound to a high affinity optimized ligand (PDB 2O2N) have been reported (Bruncko et al. 2007). Upon comparison of these two structures, it is notable that the binding site on Bcl-X_L has undergone considerable adaptation as the structure of the ligand has changed during optimization (▶Figure 17.1). As a result, the sites on Bcl-X_L occupied by the two low affinity ligands have changed considerably when compared to the structure of Bcl-X_L bound to an optimized ligand, showing that the mechanism of affinity maturation in this case does not simply involve linking two fragments in adjacent binding sites. Clearly, the protein itself adapts considerably as the ligand is changed.

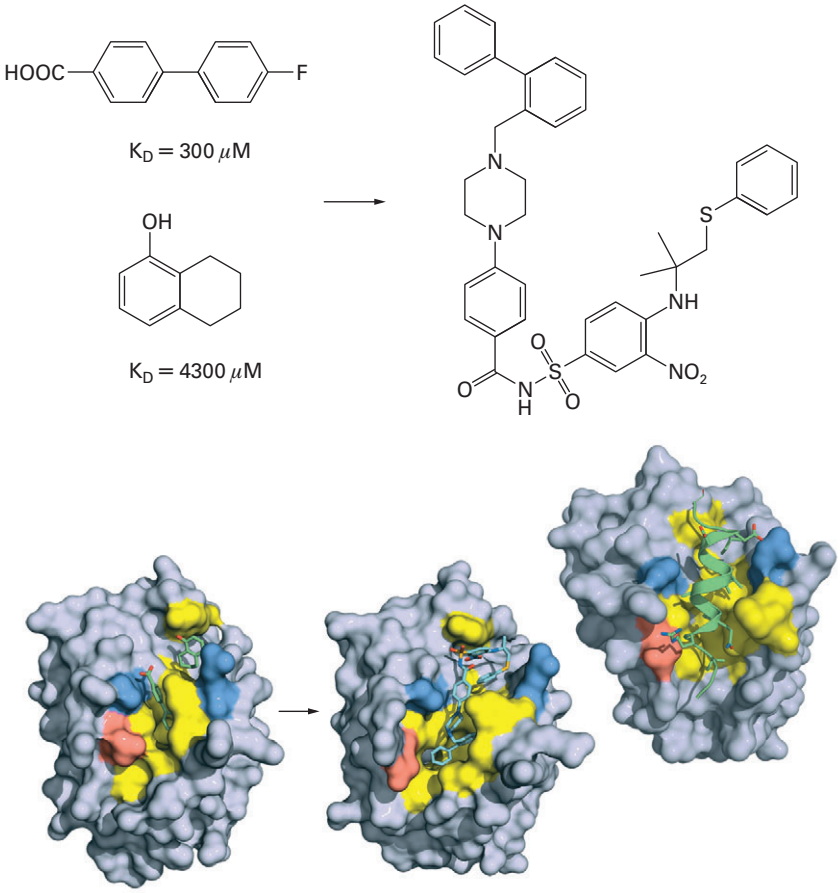

Figure 17.1: Structures of ligands bound to Bcl-x_L. A large conformational change in the protein Bcl-x_L occurs as the ligands change from the complex with low-affinity inhibitors (left, PDB 1YSG), to that with a high affinity ligand (middle, PDB 2O2N). The protein conformation is again different when bound to a helical peptide derived from an endogenous BH3 binding partner (right, PDB 1BXL). The small-molecule ligands do not mimic the structure of the peptide ligand, but rather trap the protein in a different conformation, binding in deeper cavities with more puckered grooves.

Another example of adaptivity in a protein-binding site has been observed during the discovery and optimization of an IL2-receptor-α (IL-2Rα) antagonist. A series of acylphenylalanine derivatives was discovered in an attempt to mimic the R38–F42 loop region of IL-2, one of which was found to be a competitive inhibitor of IL-2/IL-2Rα binding with an IC_{50} of 3 μM (Tilley et al. 1997) (▶Figure 17.2). Although the acylphenylalanine derivatives were designed to complex with IL-2Rα by emulating residues R38 and F42 of the IL-2 ligand, they were instead shown to interact with the ligand IL2. From this starting point, new molecules that bind to IL-2 were optimized, with dissociation constant in the mid-nanomolar range. These molecules were assembled in a fragment-based approach, guided by X-ray structures and medicinal chemistry (Arkin et al. 2003; Braisted et al. 2003; Raimundo et al. 2004). Although the small molecules were assembled before the structure of the IL-2–IL-2Rα complex had been reported (Rickert et al. 2005), they bind close to the center of the receptor contact region on IL-2. The contact epitope for the small molecule is about half the size of that for the receptor, but the small molecule and the receptor bind to IL-2 with nearly equivalent affinities. The contact surface on IL-2 for binding to IL-2Rα is relatively flat. By contrast, the small molecule traps a conformation of IL-2 in which a groove is present for small-molecule binding, and in which a loop of IL-2 has been repositioned to embrace the furanoic acid moiety at one end of the small molecule (▶Figure 17.2). Alanine-scanning mutational studies show that the small molecule and the IL-2Rα bind to the same hotspot residues on IL-2 (Thanos et al. 2006). Although the structures of the small molecule and IL-2Rα differ markedly, the electrostatic potential of the surfaces presented is similar and probably reflects a need to establish the electrostatic complementary principle with IL-2. Electrostatic and surface-shape complementary principle, as well as specific hydrogen-bonding interactions, probably account for the high selectivity of these interactions.

These studies show that the binding surface on IL-2 is adaptive and can bind to a small molecule with high affinity (K_i = 60 nM) using the same main hot-spot residues used for its natural receptor, but again without the benefit of an "O-ring." It is notable that the design of this series of IL-2-binding small molecules did not require knowledge of the structure of the bound receptor complex. Instead, the design was informed by fragment-binding data and by structures of compounds bound to IL-2, coupled with medicinal chemistry and structure–activity relationships (SAR). The small-molecule ligand is not an accurate atomic mimic of the receptor, and it would not have been discovered if it had been assumed that the precise structure of the receptor-bound form of IL-2 needed to be captured. Indeed, the adaptive nature of this protein-protein interface underscores again the challenge of applying structure-based strategies that cannot accurately predict the dynamic nature of the protein surface (Arkin et al. 2003).

17.4 Protein epitope mimetic approaches

Where 3D structural information is available for PPIs, the design of conformation constrained epitope mimetics offers one rational approach to PPI inhibitors. Molecular recognition involving proteins is typically mediated by surface exposed secondary structure elements such as ß turns, ß strands, ß hairpins and α helices. New protein-binders may be found by devising ways to mimic these important secondary structure elements in smaller "semirigid" molecules. Once interesting hits have been identified, their properties can be optimized by exploiting the fact that epitope mimetics tend to

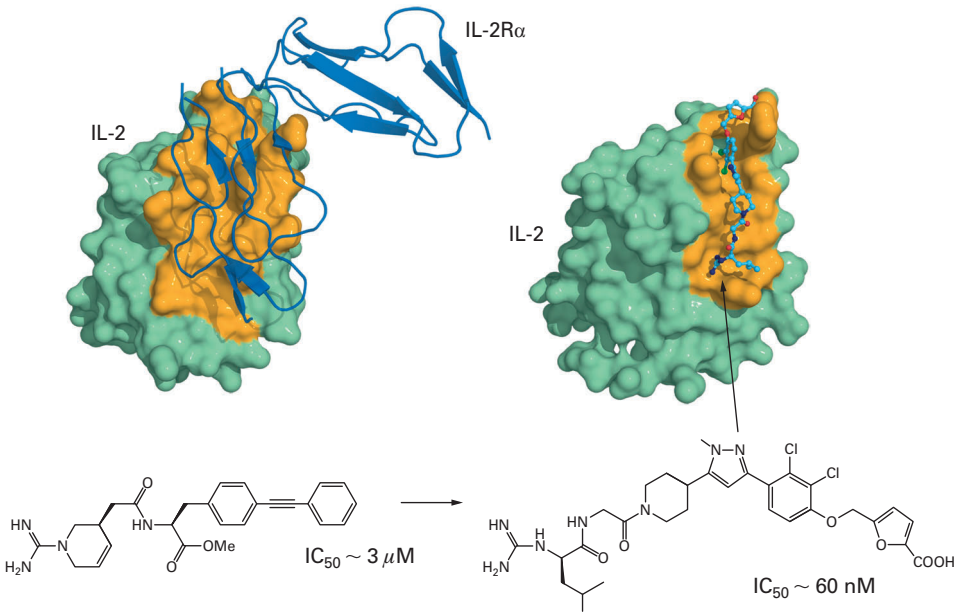

Figure 17.2: Inhibitors of the IL-2–IL-2Rα interaction. The complex of IL-2 (surface representation) and the IL-2Rα chain (ribbon) is shown left, with hot-spot residues on IL-2 shown yellow. The acylphenylalanine (left) was optimized to give the high affinity inhibitor (right) shown bound to IL-2. The small molecule traps a conformation of IL-2 in which a binding-groove is present and in which a loop of IL-2 has been repositioned to embrace the furanoic acid moiety at one end of the small molecule. Alanine-scanning mutagenesis shows that the small molecule and the receptor bind to the same hotspot residues at an adaptive binding surface on IL-2.

be modular in structure. They are typically constructed from building blocks, such as α- or ß-amino acids or peptoids, which can be linked together using robust and efficient methods. By exchanging building blocks, the mimetic structures can then be varied and properties can be optimized, in a combinatorial fashion, using parallel synthetic chemistry. One key property, essential for targeting intracellular PPIs, is permeability across the cell membrane. Progress has also been made recently in identifying cell permeable peptidomimetic PPI inhibitors, suggesting that it may be possible to engineer this property into appropriate peptidomimetic scaffolds.

17.4.1 Helix mimetics

The design of conformationally restrained mimetics of α-helical epitopes continues to attract much attention (Garner and Harding 2007; Henchey et al. 2008). Some of the approaches used so far to stabilize helical conformations in peptides include the use of intramolecular hydrogen-bond surrogates, such as hydrazone or alkenyl links (Cabezas and Satterthwait 1999; Chapman et al. 2004; Henchey et al. 2010; Patgiri et al. 2008; Vernall et al. 2009; Wang et al. 2005, 2006, 2008), and helical end-capping groups (▶Figure 17.3) (Austin et al. 1997; Kemp and Curran 1988a, 1988b; Kemp and

Rothman 1995a, 1995b; Kemp et al. 1995a, 1995b, 1996; Lewis et al. 1998; Maison et al. 2001; Obrecht et al. 1999; Schneider and DeGrado 1998). Other methods to enhance helicity in peptides include incorporating unnatural amino acids (Andrews and Tabor 1999), in particular, α-disubstituted amino acids such as Aib (Venkatraman et al. 2001) and other α-alkylated-α-amino acids.

Another approach for the stabilization of α-helical conformations in peptides is by side-chain cross-linking, also called "stapling,," of *i* and *i*+4 or *i* and *i*+7 side chains lying on the same face of the helix. The earliest reports of cross-linking involved lactam (Felix et al. 1988; Geistlinger and Guy 2003; Judice et al. 1997; Mills et al. 2006; Osapay and Taylor 1992; Phelan et al. 1997; Shepherd et al. 2006; Sia et al. 2002; Taylor 2002), disulfide (Harrison et al. 2010; Jackson et al. 1991; Leduc et al. 2003) and thioether (Brunel and Dawson 2005) bridges, as well as metal-mediated chelation (Beyer et al. 2004; Ghadiri and Choi 1990; Kelso et al. 2003, 2004; Ruan et al. 1990). Hydrocarbon bridges made using olefin metathesis chemistry have also been reported (Blackwell and Grubbs 1998). This concept has been taken a step further by combining within the same amino acids the side-chain cross-link and backbone α-methylation (Schafmeister et al. 2000). In this approach, (*R*)- and (*S*)-α-methyl-α-alkenyl amino acids are incorporated at *i* and *i*+7 positions, respectively, and then linked by ruthenium-catalyzed ring-closing metathesis (▶Figure 17.3). The cross-link results in a stabilized helical conformation in the peptide and improved stability of the peptide toward proteolysis. Several applications

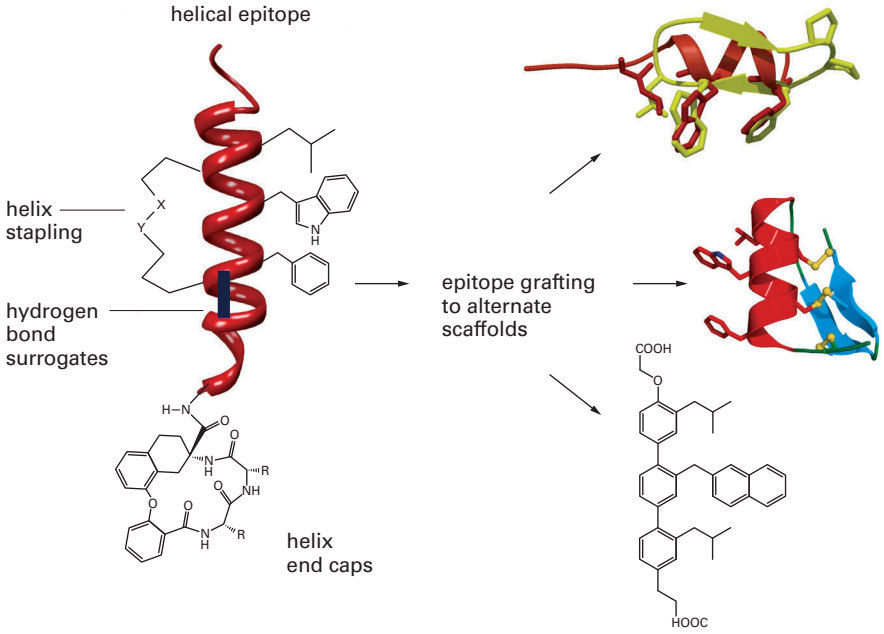

Figure 17.3: Strategies to stabilize or mimic α-helical epitopes. Helical conformations can be stabilized in linear peptides, for example, by helix-stapling, by incorporating hydrogen-bond surrogates, or by adding end-capping groups. Helical epitopes can also be grafted onto new scaffolds, including peptide-based and nonpeptidic scaffolds.

of this technology for helix mimicry have been reported in drug discovery. The conformational constraints imposed by this type of helix cross-linking have favorable effects not only on target binding affinity and proteolytic stability, but surprisingly, also on cell membrane permeability. In one example, a hydrocarbon-stapled peptide was designed to mimic an amphipathic helix in the pro-apoptotic BH3 domain of the BCL-2 family member called BID. The resulting stapled peptide showed not only a much higher helical content in water, compared to a linear control peptide, but also a higher protease stability and a 6-fold higher binding affinity to BCL-2 (39 vs. 269 nM). Moreover, the stapled peptide was apparently taken up by cells in an energy-dependent pinocytotic pathway, where it localized with its target at the outer mitochondrial membrane. The unmodified control peptide was completely impermeable to the intact cells. The stapled peptide mimic of the BH3 domain also specifically activated the apoptotic pathway to kill leukemia cells, and effectively inhibited the growth of human leukemia xenografts in vivo (Walensky et al. 2004). In other examples, a stapled BID BH3 helix was shown to bind and activate BAX, a multidomain BCL-2-family protein that resides in the cytoplasm (Walensky et al. 2006). And a stapled peptide derived from p53 was shown to bind to HDM2, penetrate cells by an endocytic transport mechanism, and reactivate apoptosis in HDM2-overexpressing cells (Bernal et al. 2007). Transcription factors have also been rather difficult to target using small drug-like molecules. However, a hydrocarbon-stapled peptide was shown to mimic MAML1 and bind directly to the oncogenic transcription factor NOTCH1. Treatment of leukaemic cells with the stapled peptide resulted in genome-wide suppression of NOTCH-activated genes (Moellering et al. 2009). These promising results, targeting a range of difficult protein-protein interactions, suggest that such stapled α-helical peptide domains may be widely useful in chemical biology, as tools for biological studies, and potentially also as drug candidates. Further work will be required to assess their pharmacological properties and toxicity, as well as their target affinities and selectivities.

Apart from peptide stapling, α-helical epitopes have been mimicked using other scaffolds, including peptoids (Vaz and Brunsveld 2008), ß hairpins (Fasan et al. 2006; Moehle et al. 2007; Seitz et al. 2010), ß-peptides and various cyclic scaffolds (Oguri et al. 2005, 2006), bi- and ter-phenyls (Ernst et al. 2002; Jacoby 2002; Kutzki et al. 2002; Orner et al. 2001) and related heterocycles (Campbell et al. 2010; Cummings and Hamilton 2010; Lee et al. 2011; Saraogi et al. 2010) and templates (Schneider and Kelly 1995).

Stable miniprotein domains also provide a source of scaffolds for epitope grafting and mimetic design (Vita 1997; Vita et al. 1998). One interesting example is the family of small naturally occurring disulfide cross-linked peptides belonging to the short-chain toxins from scorpion venom, such as charybdotoxin and scyllatoxin (Drakopoulou et al. 1996; Vita et al. 1995, 1999; Zinn-Justin et al. 1996). These toxins typically contain a short α-helical segment at the N-terminal end, cross-linked by three disulfide bridges to a ß-hairpin motif (▶Figure 17.4). Both the α-helical segment and the ß hairpin provide conformation stable segments onto which foreign epitopes can be grafted. For example, the ß-hairpin motif has been exploited to generate mimics of an epitope on CD4, the primary cellular receptor for HIV-1. HIV-1 entry into cells is initiated by the binding of the viral glycoprotein gp120 to CD4 on host cells. Crystallographic studies have shown that a key component of the epitope on CD4 that interacts with gp120 is a surface ß-hairpin loop (the CDR2-like loop) (Kwong et al. 1998). Transplanting this hairpin epitope from CD4 onto the scorpion toxin scaffold afforded, after optimization, mimetics

that bind tightly to gp120 and inhibit HIV-1 entry into cells (Huang et al. 2005; Martin et al. 2003; Stricher et al. 2008; Vita et al. 1999).

The same scorpion scaffold was also used in a different way, to generate a mimetic of the α-helical segment of p53 that interacts with its cellular inhibitor HDM2 (Li et al. 2008). This study exploited the α-helical segment of the scorpion toxin scaffold. Thus, the residues along one face of the scorpion toxin helix were replaced with the topologically equivalent residues in p53 (F19, L22, W23 and L26), such that the key hydrophobic side chains required for interaction with HDM2 are displayed in the correct relative orientation (▶Figure 17.4). With some additional optimization, a p53 mimetic based on this toxin scaffold was obtained that bound to HDM2 with submicromolar affinity. The question of cellular membrane permeability was also addressed in this study (Li et al. 2008). In order to enhance cell permeability, the mimetic was reengineered by replacing five residues near the C terminus with arginines, to create a cluster of eight cationic residues. This gave a molecule that, when added to a $p53^+$ cancer cell line, was able to kill cells quantitatively and in a p53-dependent manner, apparently due to its ability to traverse the cell membrane and inhibit the p53-HDM2 interaction.

Another small folded peptide that has been used for engineering experiments is the avian pancreatic polypeptide (aPP) (Hodges and Schepartz 2007). aPP contains 5 turns of α helix in its C-terminal half, linked to a 10-residue extended (ß structure) N-terminal segment, which is back-folded onto the α helix. The p53 epitope has also been successfully grafted onto this aPP scaffold, to create miniprotein inhibitors of the p53-MDM2 interaction (Kritzer et al. 2006). The cyclotides and conotoxins represent two other families of macrocyclic cross-linked peptides, each with great potential as scaffolds in protein epitope mimetic design (Cascales and Craik 2010; Clark et al. 2010; Craik et al. 2006a, 2006b; Green et al. 2007; Henriques and Craik 2010).

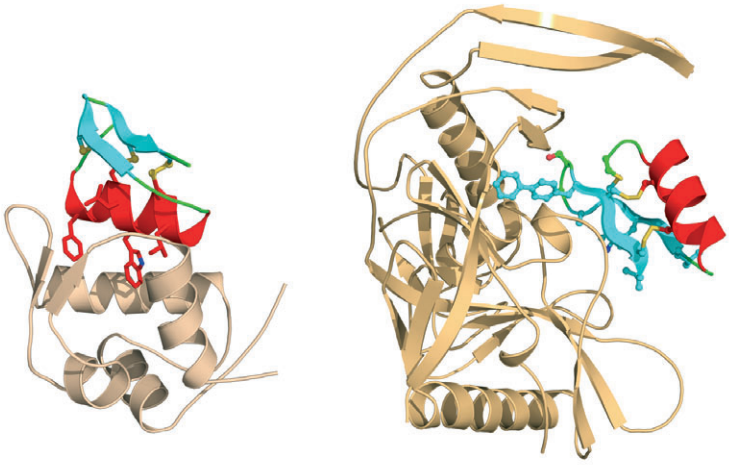

Figure 17.4: Both α-helical and ß-hairpin epitopes have been grafted onto the scorpion toxin scaffold, resulting in mimetics of p53 for binding to HDM2 (shown left) and of CD4 binding to gp120 from HIV-1 (shown right).

A more recent trend is toward the design of novel folding architectures, or "foldamers," that adopt regular structural elements akin to α helices and ß structure, but built using other types of building blocks (i.e. not α-amino acids) (Goodman et al. 2007; Guichard and Huc 2011; Hill et al. 2001; Smith et al. 2011). Stable helix-like conformations have been characterized in oligomers prepared from N-alkylglycines (peptoids) (Fowler and Blackwell 2009) and from ß- and γ-amino acids (Horne and Gellman 2008; Seebach et al. 2004). The ß-peptides have been the most studied family of peptidomimetic foldamers, but whereas many short ß-peptides adopt well-defined conformations in organic solvents, additional helix-stabilizing elements (e.g. ring constraints or helix stapling) are required for stable folds to occur in aqueous solution (Appella et al. 1999; Vaz et al. 2008). Aromatic and aliphatic *N,N'*-linked oligoureas have also been designed to fold and/or self-assemble in a controlled manner (Fischer and Guichard 2010). The development of foldamers with heterogeneous backbones formed by combining multiple residue types (mixed α/ß-peptides, oligo-urea/amides) has also been reported (Claudon et al. 2010; Horne and Gellman 2008).

Some of these novel foldamer scaffolds have also been applied successfully to PPI inhibitor design. For example, ß-amino acids have been incorporated into a mimic of the helical coiled-coil heptad repeat region 2 (HR2) of gp41 from HIV-1. This HR2 region must interact with the HR1 heptad repeat region before fusion of the viral and target cell membranes can occur. The chimeric α/β-peptides were shown to mimic structural and functional properties of the critical α-helical HR2 epitope in gp41. Biophysical and crystallographic studies, and results from cell-fusion and virus-infectivity assays, collectively indicate that the gp41-mimetic α/β-peptides effectively block HIV-cell fusion via a mechanism comparable to that of gp41-derived α-peptides such as T20 (Fuzeon) (Horne et al. 2009). In a related study, a series of ß3-decapeptides, also based on gp41, were designed with a ß-peptide 14-helical conformation stabilized by macro dipole neutralization and side chain-side chain salt bridges (Stephens et al. 2005). The peptide mimetics were shown to inhibit syncytia formation in cell culture, again by blocking PPIs important for infection by HIV-1. A similar viral-host cell fusion mechanism is also exploited by other viruses (Eckert and Kim 2001). Thus, helical ß-peptides have been designed that target in a similar way the coiled-coil PPIs that occur during infection by human cytomegalovirus (English et al. 2006).

ß-Peptides have also been designed to mimic helical epitopes in the tumor suppressor protein p53 and in antiapoptotic BCL-2 family members such as BH3. For example, salt bridge-stabilized 14-helical ß-peptides have been described, bearing on one face of the helix residues required for binding to MDM2 (Kritzer et al. 2004). The most potent ß-peptides were reported to bind MDM2 and MDMX, and inhibit the p53-MDM2/MDMX PPI, with nanomolar affinities in a direct fluorescence polarization assay (Michel et al. 2009). In later studies, ß-peptides with hydrocarbon and diether staples (bridges) were prepared that not only potently inhibit the p53-MDM2 interaction, but also are taken-up by mammalian cells far more efficiently than unbridged analogues (Bautista et al. 2010). In the case of the BH3/Bcl-x_L interaction, sequence rather than structural information on the interaction of BH3 domains with Bcl-x_L has been exploited to design inhibitors based on chimeric α/ß-peptides (Horne et al. 2008). The approach involved replacing subsets of regularly spaced α residues with β3 residues bearing the original side chains. Each α/β-peptide contains an ααβαααβ backbone repeat, which is derived from the heptad pattern common among α-peptide sequences that form α

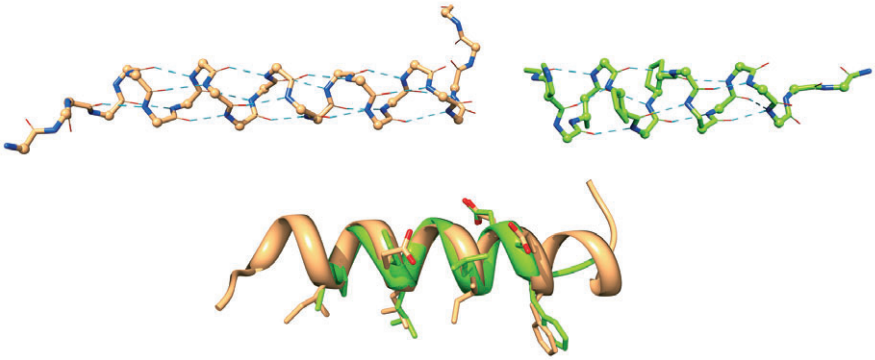

Figure 17.5: Chimeric α/ß-peptide (right) designed to mimic an α-helical epitope (left) in the BH3 domain for binding to Bcl-x_L.

helices with a well-developed "stripe" of hydrophobic side chains running along one side. This approach led to α/ß-peptides with comparable binding affinity for the protein target and substantially improved proteolytic stability. A crystal structure of a complex between one α/ß-peptide foldamer and the protein partner Bcl-x_L shows that the foldamer adopts a helical conformation and is oriented within the BH3 recognition groove in a similar fashion to natural BH3 domains (Lee et al. 2009) (▶Figure 17.5). The C-terminal α-peptide portion is α helical, whereas the α/ß segment adopts a characteristic 14/15-helical structure with some cyclic ß-amino acid residues (*trans*-2-aminocyclopentane carboxylic acid) making contacts with the protein surface. The helix formed by the Bcl-x_L-bound foldamer features a network of backbone C=O(*i*)···H-N(*i*+4) H-bonds over nearly the entire length of the oligomer. The crystal data suggest that the foldamer achieves high affinity in part by mimicking the three-dimensional display of the canonical side chains projected by natural BH3 domains, however, the β-residue contacts may also contribute significantly to foldamer affinity.

17.4.2 ß-Hairpin mimetics

ß structure and in particular ß hairpins also occur frequently in protein-protein recognition epitopes. Not surprisingly, therefore, the design of hairpin mimetics has also attracted great interest. ß-Hairpin loops are found in the antigen-binding sites of antibodies and the ligand binding sites of many cytokine receptors and polypeptide growth factors, as well as in many integrins and viral proteins, a wide variety of smaller host-defence peptides such as defensins, venom toxins such as ω-conotoxin and three-finger snake toxins, in cyclotides, and in Bowman-Birk and related proteinase inhibitors, to name but a few. Naturally occurring ß-hairpin motifs possess a remarkable degree of structural diversity, due to variations in loop size, variations in the hairpin register, due to the occurrence of ß-bulges within the ß strands, and of course due to variations in sequence. The register of a ß hairpin defines which pairs of cross-strand amino acids occupy hydrogen-bonding (HB), rather than non-hydrogen-bonding (NHB), positions. This in turn determines which amino acid side chains are displayed on the same face of the hairpin (▶Figure 17.6).

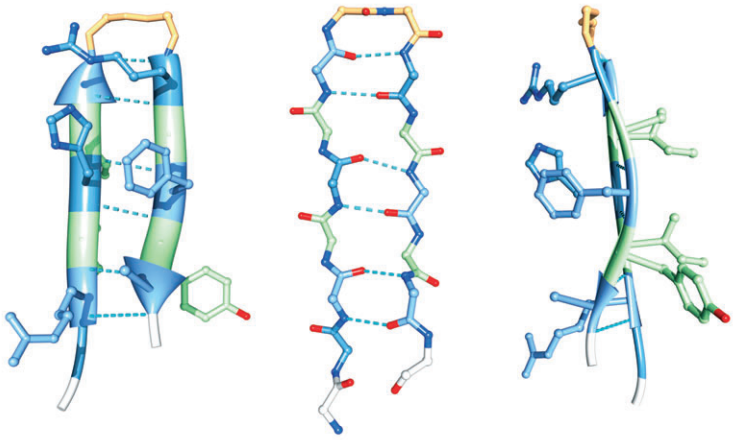

Figure 17.6: The register of a ß-hairpin loop defines which pairs of cross-strand amino acids occupy hydrogen-bonding (HB) (indicated with blue dashed lines) and NHB.

ß-Hairpin loop structures can be stabilized in linear peptides using disulfide bridges. This approach has been particularly valuable in phage-display technology to select peptides that bind to specific protein targets (Sidhu et al. 2000). Spectacular examples have been reported of disulfide-bridged hairpin-loop peptides selected by phage display that can mediate (agonist) or inhibit PPIs. For example, by screening a random phage library against the immobilized erythropoietin receptor (EPO-R), a disulfide-bridged peptide [GGTYSCHFGPLTWVCKPQGG] was found, that binds to EPO-R in a ß-hairpin conformation (▶Figure 17.7) (Livnah et al. 1996; Wrighton et al. 1996). This hairpin peptide mimics the helical cytokine EPO by interacting with the EPO-binding site on the receptor, causing dimerization of the extracellular domain. As a result, both EPO and the peptide induce a similar signalling cascade of phosphorylation events and cell cycle progression in EPO responsive cells. In another example, a phage-library of cyclic peptides was screened for binding to the constant fragment (Fc) of immunoglobulin G (IgG) (DeLano et al. 2000). A disulfide bridged hairpin peptide was isolated that interacts with the Fc at a highly accessible adaptive and hydrophobic site that also is used by other Fc-binding proteins, including protein A, protein G, rheumatoid factor and the neonatal Fc-receptor. Phage display is now a well-established and powerful approach for selecting peptides and proteins with novel binding functions from large combinatorial libraries (Sidhu et al. 2003).

A disulfide bridge, however, has disadvantages, because it can be cleaved in vivo by reaction with a free thiol group, and the disulfide link has many degrees of rotational freedom, which in the absence of other stabilizing interactions (Russell et al. 2002), can make it difficult to stabilize discrete hairpin registers or conformations. ß-Hairpin conformations have been successfully stabilized in linear peptides by exploiting tryptophan zipper motifs (π-π-stacking interactions between cross-ß-strand tryptophans at NHB positions), and related interactions (Eidenschink et al. 2009; Russell et al. 2002; Wu et al. 2010b). The residues in the turn region have a strong influence upon ß-hairpin

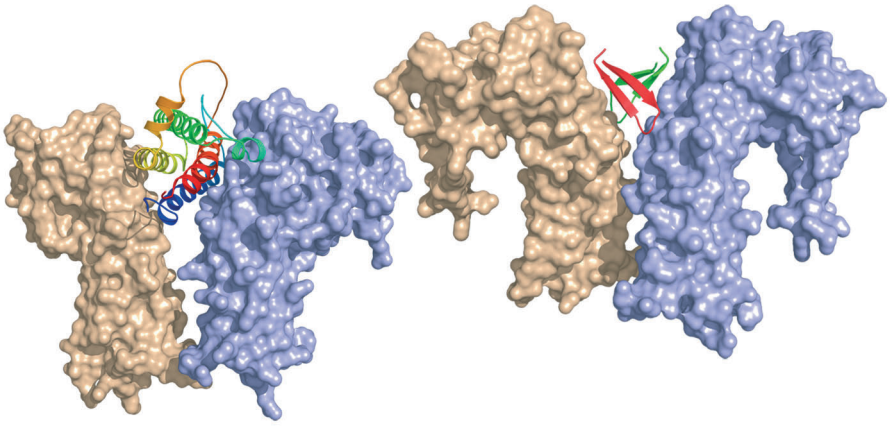

Figure 17.7: A disulfide bridged ß-hairpin peptide selected by phage display (right) mimics the helical cytokine EPO (left) in binding to and activating the EPO-receptor.

stability in linear peptides. Turns formed by Asn-Gly, D-Pro-Gly, Aib-Gly and Aib-D-Pro are strong promoters of ß-hairpin formation due to their propensity to form type-I′ or -II′ turns (Raghavender et al. 2010; Rai et al. 2007), which match the preferred right-handed twist of a hairpin. Other building blocks have been incorporated into turn regions and into the ß-strand regions, including, for example, the 1,2-dihydro-3(6H)-pyridinyl unit that favors extended conformations (Phillips et al. 2005). A novel hairpin-capping motif was described recently, which appears to overcome the problem of fraying at the ends of the ß strands in hairpin motifs in linear peptides (Kier et al. 2010). The capping motif comprises synergistic stacking and hydrogen-bonding interactions between an N-terminal alkanoyl-Trp and a C-terminal -TrpThrGly- motif, which effectively tie together the ß strands as long as the Trp residues are at terminal NHB positions.

Stable ß-hairpin scaffolds can also be achieved by linking together the N and C termini, to produce macrocyclic hairpin structures. Nature has also exploited this strategy, as evidenced by macrocyclic natural products such as gramicidin S, which has a hairpin structure with two D-Phe-L-Pro units marking the turn regions. Other approaches to macrocycles with stable parallel ß-sheet scaffolds have also been described recently (Freire and Gellman 2009; Woods et al. 2007).

Alternatively, ß-hairpin structures in naturally occurring peptides and proteins can be mimicked by transplanting the hairpin loop onto an appropriate (semi)-rigid template; the so-called protein epitope mimetic (PEM) technology (Robinson 2008; Robinson et al. 2008). The dipeptide D-Pro-L-Pro has proved to be an extremely useful template in ß-hairpin mimetic design (▶Figure 17.8). This dipeptide adopts a rigid type-II′ ß turn, which is ideal to initiate antiparallel strands held together by registered cross-strand hydrogen bonds. Upon transplanting the loop from the protein of interest, the cross-strand residue pair directly attached to the D-Pro-L-Pro template then occupay a hydrogen-bonding position. In this way, accurate structural mimetics have been produced of CDR loops found in antibodies (Favre et al. 1999), of a ß-hairpin loop in Tat bound to HIV-1 TAR-RNA (Athanassiou et al. 2004, 2007; Davidson et al. 2009; Lalonde et al. 2011; Leeper et al. 2005), of protease inhibitors related to the Bowman-Birk family (Descours

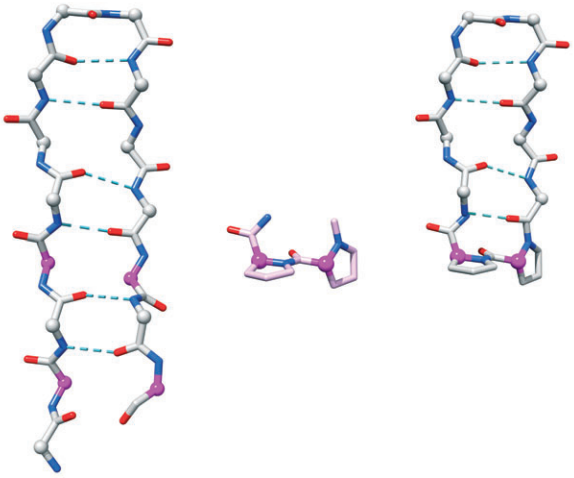

Figure 17.8: ß-Hairpin mimetics can be produced by transplanting the hairpin loop from a protein of interest (left), onto a hairpin-stabilizing template, such as the dipeptide D-Pro-L-Pro (center), to give a cyclic ß-hairpin PEM (right).

et al. 2002), and of cationic host-defence peptides related to protegrin-I (Robinson et al. 2005; Shankaramma et al. 2002, 2003; Srinivas et al. 2010) and polyphemusin (DeMarco et al. 2006). In another example, a 13-residue disulfide cross-linked loop taken from a phage display peptide that binds a human antibody Fc fragment (discussed previously in this section) (DeLano et al. 2000) was transplanted onto a D-Pro-L-Pro template (Dias et al. 2006). The resulting 13-mer loop was shown to adopt a well-defined ß-hairpin structure, which includes a bulge in the second strand close to the turn region, with the unusual result that side-chains of two adjacent residues point to the same side of the hairpin.

The interest in ß-hairpin mimetics is enhanced further by the realization that a hairpin scaffold can also be exploited to mimic α-helical epitopes. Thus, the distance between the Cα atoms of two residues *i* and *i*+2 along one strand of a hairpin is very close to that between the Cα atoms of two residues *i* and *i*+4 on one face of an α helix. Using this information it was possible to design a ß hairpin to mimic the pharmacophore within a helical peptide derived from p53 (▶Figure 17.9), which is involved in binding to its interacting protein HDM2 (Fasan et al. 2004, 2006). Template-bound ß-hairpin mimetics were found that bind with nanomolar affinity to HDM2 (Grässlin et al. 2009). A crystal structure of one mimetic bound to HDM2 confirmed that the hairpin scaffold presents three side chains along the first strand of the hairpin for binding to HDM2, just like the corresponding three residues situated on one face of the helical p53 (▶Figure 17.9). In addition, residues in the second ß strand of the mimetic make additional favorable contacts with HDM2, which are not seen in the p53-HDM2 complex. Other examples of hairpin mimicry of helical epitopes have been reported more recently, based on the interactions of Rev protein to RRE-RNA, and of a helical epitope in the chemokine receptor CCR5 that binds to the HIV-1 glycoprotein gp120 (Moehle et al. 2007; Seitz et

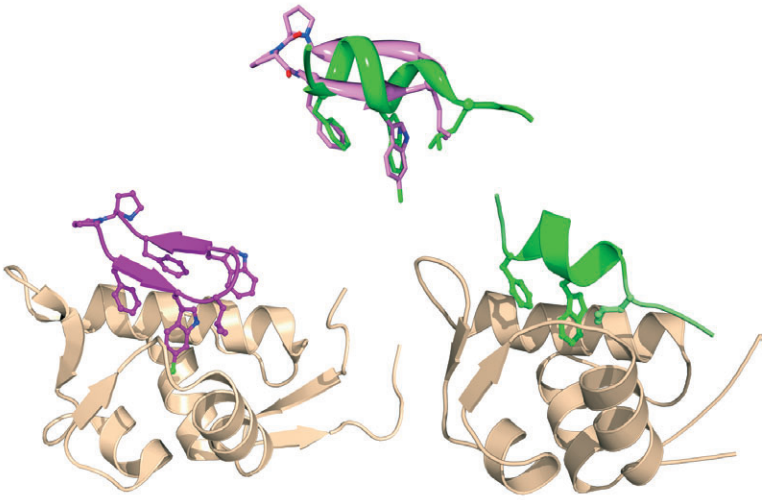

Figure 17.9: ß-Hairpin mimetics can also be used to mimic the α-helical epitope in p53. A crystal structure of one hairpin mimetic bound to HDM2 is shown (left). The cyclic peptide scaffold is used to display hot-spot residues lying along one face of an α-helical epitope in p53, which are important for binding to HDM2 (right).

al. 2010). These examples suggest that the hairpin scaffold might be widely applicable in the design of helical epitope mimetics.

The macrocyclic ß-hairpin mimetics described previously in this section are constructed from building blocks that can be linked together using robust and efficient solid-phase synthesis methods. By exchanging building blocks, the mimetic structures can be quickly varied and their properties optimized, in an efficient combinatorial fashion, using parallel synthetic chemistry (Jiang et al. 2000). The ease with which analogues can be produced in this way, contrasts with the difficulties that often exist in synthesizing analogues of complex natural products of similar size. The variables for creating combinatorial libraries include the size of the ß-hairpin loop, the types of amino acid building blocks, as well as the template (▶Figure 17.10). The proteinogenic α-amino acids often make convenient starting points for mimetic design, but may be exchanged for any of a large number of known nonproteinogenic amino acids, or related building bocks. This provides great scope for optimizing drug-like properties (ADMET), as well as target affinity and specificity, within a lead series. This point is illustrated by recent examples that have led to clinical drug candidates (Obrecht et al. 2011; Robinson et al. 2008). In one case, a family of PEM molecules modeled on the naturally occurring cationic antimicrobial peptide protegrin I (PG-I) was discovered using the PEM technology. The lead compound **L27–11** is a potent antibiotic with a novel mechanism of action, which is active in the nanomolar range against Pseudomonas species and, in particular, the important human pathogen *Pseudomonas aeruginosa* (Srinivas et al. 2010). This family of PEM molecules does not lyse cells, but rather, they are able to target a bacterial ß-barrel outer membrane (OM) protein called LptD, which is essential for the assembly of the outer leaflet of the OM in many gram-negative bacteria (Chng et al. 2010; Ruiz et al. 2009; Sperandeo et al. 2009). When the function of LptD is blocked,

OM biogenesis is disrupted. The discovery of **L27–11** involved an iterative process of library synthesis and screening, in which an initial hit was identified by PEM library synthesis and screening for antimicrobial activity. The optimal hit from each library was used as a starting point for the synthesis and testing of variations in a subsequent library. The same procedure was also followed to optimize the drug-like properties of **L27–11**, including the plasma stability, target selectivity, and toxicology. This process resulted in the clinical lead candidate **POL7080** (Srinivas et al. 2010). The safety of mimetic **POL7080** is now being tested in healthy humans in a phase I clinical study, but it is clear already that this mimetic represents the first in a new class of antibiotics active against gram-negative bacteria.

A second example is provided by a family of PEM molecules that were based upon the naturally occurring peptide polyphemusin II, an 18-amino-acid peptide isolated from the American horseshoe crab (*Limulus polyphemus*), which inhibits the chemokine and G-coupled protein receptor CXCR4. Based on the solution structure of polyphemusin II, several PEM molecules were designed and optimized in biological assays. This led to highly potent and selective CXCR4 antagonist such as **POL3026**, **POL5551** and **POL6326** (DeMarco et al. 2006; Robinson et al. 2008). **POL6326** has now successfully moved into a phase II clinical trial for autologous stem cell transplantation in newly diagnosed multiple myeloma patients. Interim results from this study show that **POL6326** is safe, well tolerated and efficient in mobilizing hematopoetic stem cells. Recently, an

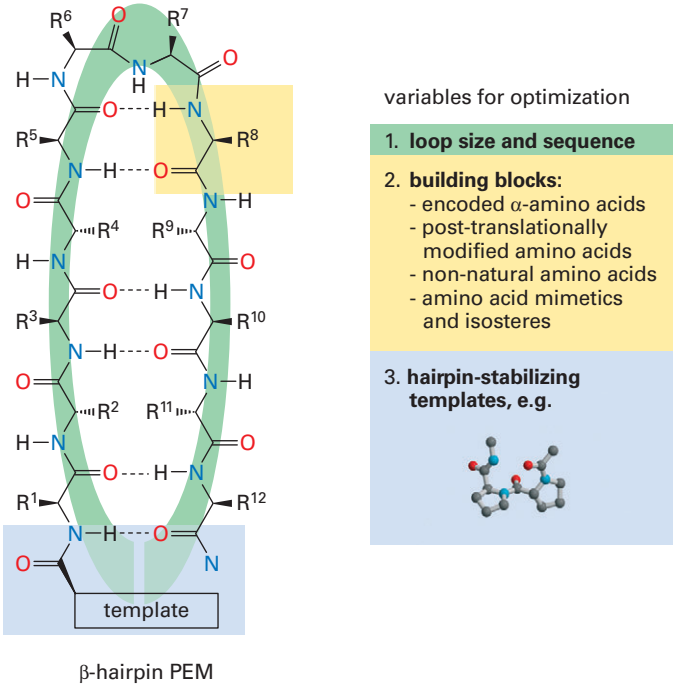

Figure 17.10: The structures and properties of ß-hairpin protein epitope mimetics (PEMs) can be varied and optimized through library synthesis and screening. Some of the variables for library design are indicated.

X-ray structure revealed how a very closely related ß-hairpin-shaped peptide binds to an engineered form of CXCR4 (Wu et al. 2010a). The ß hairpin occupies a binding site on CXCR4 formed by residues in the inward-facing protruding walls of the seven transmembrane helical bundle, several extracellular loops, and the N-terminal segment. A network of polar, hydrogen-bonding and hydrophobic contacts between the ligand and the receptor are responsible for the specific high affinity interaction. The close structural similarity between this bound ligand and **POL3026**, suggests that the macrocyclic ß-hairpin mimetic should interact with CXCR4 in a similar manner.

The genomic and proteomic revolutions continue to provide us with an ever-increasing number of mechanistic insights into biological signalling pathways, and potential targets for inhibition using small molecules. Unfortunately, many of the genes and gene products that are often most attractive from a biological perspective for targeting using small molecules are also often the least tractable from the perspective of small drug-like molecules. Such targets typically include proteins that participate in PPIs or protein-nucleic acid interactions, which are often deemed to be "undruggable" within major pharmaceutical companies (Arkin and Wells 2004; Hopkins and Groom 2002). The difficulty in addressing "undruggable" targets using small drug-like molecules (Wells and McClendon 2007) provides a powerful motivation to explore new scaffolds and new regions of molecular space to overcome this problem. The protein epitope mimetics described in this article are positioned within a huge and relatively unexplored region of molecular space, having a size and complexity between that of traditional small drug-like molecules and much larger 'biologics', such as antibodies. It is clear already that protein epitope mimetics can provide a rich source of interesting leads for use in chemical biology, as well as drug and vaccine research. And the scope for further innovation and discovery in this field appears to be immense.

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